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Novel Proteins That Modulate Type IV Pilus Retraction Dynamics in *Pseudomonas aeruginosa*▽

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Pseudomonas aeruginosa **uses type IV pili to colonize various materials and for surface-associated twitching motility. We previously identified five phylogenetically distinct alleles of *pilA* in *P. aeruginosa*, four of which occur in genetic cassettes with specific accessory genes (J. V. Kus, E. Tullis, D. G. Cvitkovitch, and L. L. Burrows, Microbiology 150:1315–1326, 2004). Each of the five pilin alleles, with and without its associated pilin accessory gene, was used to complement a group II PAO1 *pilA* mutant. Expression of group I or IV *pilA* genes restored twitching motility to the same extent as the PAO1 group II pilin. In contrast, poor twitching resulted from complementation with group III or group V *pilA* genes but increased significantly when the cognate *tfpY* or *tfpZ* accessory genes were cointroduced. The enhanced motility was linked to an increase in recoverable surface pili and not to alterations in total pilin pools. Expression of the group III or V pilins in a PAO1 *pilA-pilT* double mutant yielded large amounts of surface pili, regardless of the presence of the accessory genes. Therefore, poor piliation in the absence of the TfpY and TfpZ accessory proteins results from a net increase in PilT-mediated retraction. Similar phenotypes were observed for *tfpY* single and *tfpY-pilT* double knockout mutants of group III strain PA14. A $PilA_V$-TfpY chimera produced few surface pili, showing that the accessory proteins are specific for their cognate pilin. The genetic linkage between specific pilin and accessory genes may be evolutionarily conserved because the accessory proteins increase pilus expression on the cell surface, thereby enhancing function.**

Pseudomonas aeruginosa is a successful opportunistic pathogen, in part due to its ability to colonize a wide spectrum of living and nonliving surfaces using its type IV pili (T4P) (6, 17, 19). The long polar T4P allow dissemination from the initial point of colonization via surface-associated twitching motility, which results from alternating pilus extension and retraction (20, 34). The T4P system of *P. aeruginosa* is complex, with over 50 gene products involved directly or indirectly in pilus biogenesis, regulation of pilus expression, and chemotactic control of twitching motility, identified to date in well-studied laboratory strains such as PAO1 and PAK (34).

In *P. aeruginosa*, T4P are composed of a single type IVa pilin protein encoded by the *pilA* gene. This gene is found at a conserved chromosomal locus between the divergently transcribed *pilB* gene (encoding the pilin polymerase) and a $tRNA^{Thr}$ gene. We showed previously that there are at least five distinct alleles of *pilA* in the *P. aeruginosa* species, with group I pilins being the most prevalent type identified in our survey (29). Phylogenetic analyses showed that group I and II pilins were more closely related to one another than to group III, IV, or V pilins. Of the five *pilA* alleles, all but one (group II, the allele present in laboratory strains PAO1 and PAK) are linked with one or two characteristic accessory genes located immediately downstream of the pilin gene. Each pilin allele was strictly associated with its cognate accessory gene(s), suggesting that the pilin and accessory genes may be horizontally transferred as a genetic cassette (29).

Group I pilins are associated with the *tfpO* (*pilO*) accessory gene, encoding a pilin glycosyltransferase that posttranslationally modifies each pilin subunit on a C-terminal Ser residue with an O-antigen unit prior to assembly into the pilus (9, 11). TfpO has broad substrate specificity and will modify group I pilins with any O-antigen unit that has a dideoxyhexose sugar as the lipid-carrier-proximal moiety (14). This modification influences both the physical properties of pili and their function, as *tfpO* mutants were less virulent than the parental strain in competitive animal infection assays (45).

We recently demonstrated that pilins from the group IV strain Pa5196, which are associated with the *tfpW* and *tfpX* accessory genes, are also posttranslationally glycosylated (48). However, the glycan is not an O-antigen unit, but instead a novel homopolymer of D-arabinofuranose that is identical to those forming part of the lipoarabinomannan and arabinogalactan cell wall polymers of the *Corynebacterineae*, including *Mycobacterium tuberculosis* and *M. leprae* (48). Like TfpO, the product of the *tfpW* gene is predicted to be a large inner membrane protein with multiple membrane-spanning domains and a glycosyltransferase motif, although there is no sequence similarity between the proteins. The role of TfpW in glycosylation of group IV pilins will be presented elsewhere (29a).

The group III and group V pilins identified in our previous study were not glycosylated based on periodic acid-Schiff staining and are not associated with either *tfpO*- or *tfpW*-like genes (29). The accessory genes associated with group III and group

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V pilins are *tfpY* and *tfpZ*, respectively. These genes, as well as the related group IV accessory gene *tfpX*, are predicted to encode inner membrane proteins with approximately 50% amino acid sequence similarity to one another, concentrated in three predicted N-terminal transmembrane domains. These *P. aeruginosa* protein genes have limited sequence similarity to the pilin accessory genes *fimB*, identified in the sheep pathogen *Dichelobacter nodosus*, and *pilB*, from the human pathogen *Eikenella corrodens* (28, 29, 46).

In this study, we cloned each of the five *pilA* alleles of *P. aeruginosa*, with or without their cognate accessory genes, into *pilA* mutants of the PAO1 laboratory strain. We show that while the pilins of groups I and IV complemented twitching motility to the same extent as the cognate pilin in the PAO1 background, pilins of groups III and V did not. The provision of the *tfpY* and *tfpZ* genes enhanced twitching motility in PAO1 recombinant strains expressing group III and V pilins, respectively. This phenotype was linked to enhanced display of pili on the cell surface arising from altered pilus retraction dynamics.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and genetic constructs used for this study are listed in Table 1. Bacteria were maintained as glycerol stocks at −80°C and routinely grown on Luria-Bertani (LB) plates, supplemented where indicated with L-arabinose and antibiotics. Antibiotic concentrations for *Escherichia coli* or *P. aeruginosa* were 15 or 30 mg/liter of gentamicin (Gm), 15 or 50 mg/liter of tetracycline, and 100 mg/liter of ampicillin or 200 mg/liter of carbenicillin, respectively.

Genetic manipulations. For complementation studies, *pilA* genes, alone or with their cognate accessory genes, were amplified by PCR from chromosomal DNA of representative group I, II, III, IV, and V strains as indicated in Table 1. Primers included restriction sites EcoRI or HindIII for cloning. Sequences were as follows: for primer 1 (forward $pilA_{I}$), 5′ AAGAATTCATGAAAGCTCAGAAGGGT; for primer 2 (forward $pilA_{II}$, $pilA_{III}$, $pilA_{V}$), 5′ AAGAATTCATGAAAGCTCAAAAAGGC; for primer 3 (forward $pilA_{IV}$), 5′ AAGAATTCATGAAAGCGCAAAAAGGC; for primer 4 (reverse $pilA_{I}$), 5′ AGAAGCTTCAAAACAACTCAAAAAACC; for primer 5 (reverse $pilA_{III}$), 5′ AGAAGCTTGCCATCCTCCTGCTATTC; for primer 6 (reverse $pilA_{IV}$), 5′ AGAAGCTTAAAAAGAGACAAGCCCCGCA; for primer 7 (reverse *tfpW*), 5′ AGAAGCTTATACTGGAAAAAAGAAGATG; for primer 8 (reverse $pilA_{V}$), 5′ AGAAGCTTCTCACAACTTTCCGTCTTTT; and for primer 9 (reverse $tRNA^{Thr}$), AAAAAGCTTCGAATGAGCTGCTCTACCGACAGAGCT (EcoRI restriction sites are doubly underlined, and HindIII sites are singly underlined). Combinations of these primers were used to amplify pilin genes as follows: for $pilA_{I}$, primers 1 and 4; for $pilA_{I}$ plus *tfpO*, primers 1 and 9; for $pilA_{II}$, primers 2 and 9; for $pilA_{III}$, primers 2 and 5; for $pilA_{III}$ plus *tfpY*, primers 2 and 9; for $pilA_{IV}$, primers 3 and 5; for $pilA_{IV}$ plus *tfpW*, primers 3 and 9; for $pilA_{IV}$ plus *tfpW* plus *tfpX*, primers 3 and 9; for $pilA_{V}$, primers 2 and 8; and for $pilA_{V}$ plus *tfpZ*, primers 2 and 9. PCR products were purified using Qiaspin columns (Qiagen), digested with the appropriate enzymes, and ligated into the previously linearized pBADGr vector, a modified version of pMLBAD (30) wherein the *dhfr* gene encoding trimethoprim resistance was replaced with the *aacC1* gene from pUCGm (41), placing the genes under L-arabinose control. After confirming the fidelity of the constructs by DNA sequence analysis, they were introduced into a *pilA* mutant of PAO1 (26) via electroporation, and transformants were selected by plating on LB plates containing 30 mg/liter Gm. The QuikChange mutagenesis kit (Stratagene) was used as directed by the manufacturer to generate premature stop codons within the cloned accessory genes where indicated in Table 1.

Twitching motility assays. Twitching motility was assessed using an agar subsurface assay as described previously (42), and the resulting zones of twitching motility were visualized by carefully removing the agar and staining the bacteria adhering to the petri dish with 1% (wt/vol) crystal violet for 10 min at room temperature, followed by a brief rinse with tap water to remove unbound dye. ImageJ software (NIH) was used to measure and calculate average areas of the resulting twitching zones to acquire quantitative comparative data.

Analysis of sheared surface proteins by SDS-PAGE. Cell surface appendages (flagella and pili) were isolated using the methods of Castric (9) with modifications. Bacteria were streaked in a grid pattern on LB agar plates containing 30 mg/liter Gm and 0.2% L-arabinose (two plates per sample) and incubated overnight at 37°C. The bacteria were gently scraped from the agar surface by use of a sterile coverslip and resuspended in 2 ml sterile phosphate-buffered saline (PBS, pH 7.4) per sample, and surface proteins were sheared by vigorous vortexing for 30 s. The suspension was transferred to 2- by 1.5-ml microcentrifuge tubes and centrifuged for 5 min at maximum speed to pellet the cells. The supernatant was transferred to a new tube and centrifuged for an additional 25 min at maximum speed at room temperature to remove any remaining cells. To precipitate the sheared proteins, 1/10 volumes each of 5 M NaCl and 30% polyethylene glycol (molecular weight range, 8,000) were added to the supernatant and the samples incubated on ice for 60 min. Samples were centrifuged at maximum speed in a microcentrifuge for 25 min at 4°C. After discarding the supernatant, the resulting pellets were resuspended in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading dye (125 mM Tris, pH 6.8; 2% [wt/vol] 2-mercaptoethanol; 20% [vol/vol] glycerol; 0.001% [wt/vol] bromophenol blue; 4% [wt/vol] SDS), boiled for 5 min, and resolved on a 15% SDS-PAGE minigel with a prestained benchmark protein ladder (Invitrogen). The proteins were visualized using Coomassie blue dye. To compare the amounts of recoverable surface pilins between strains, densitometry of the pilin and flagellin bands in each lane was performed using ImageJ software (Scion). Pilin bands were compared after normalization to their corresponding flagellin bands and were reported in terms of differences compared to relevant controls.

Western immunoblot analysis of pilins in whole-cell lysates. After being vortexed to remove surface proteins, the harvested cell pellet was resuspended in sterile PBS to a final optical density at 600 nm of 0.6. A 200-μl aliquot of the cell suspension was transferred to a 1.5-ml microcentrifuge tube and the cells were harvested by centrifugation at maximum speed for 5 min. The supernatant was removed and the cell pellet was resuspended in 150 μl of 1× SDS-PAGE sample buffer and boiled for 5 min, and 8 μl per sample was separated on a 15% SDS-PAGE minigel as described above. After separation, the proteins were transferred to nitrocellulose for Western immunoblot analysis with rabbit polyclonal antibodies raised against the PilA proteins from strains Pa141123 (group III) and Pa110594 (group V) (29). Blots were blocked with 5% skim milk in PBS overnight at 4°C and then incubated with a 1/5,000 dilution of primary antibody for 60 min at room temperature, washed with PBS, and incubated with a 1/5,000 dilution of goat anti-rabbit alkaline phosphatase-conjugated secondary antibody for 60 min at room temperature. After being washed, blots were developed with 5-bromo-4-chloro-3-indolylphosphatase (BCIP)–Nitro Blue Tetrazolium (Sigma) as per the manufacturer's instructions.

Mass spectrometry analysis. For mass spectrometry of group III and V pilins, pili were isolated using NaCl/PEG precipitation of sheared surface proteins as reported previously (48). Sixty LB agar plates were used for each sample. Precipitated proteins were resuspended in 2 ml of 50 mM ammonium bicarbonate buffer, pH 8.5, and stored at −20°C. Because the PEG was found to affect the mass spectrometry results, it was removed by adding 9 volumes of ice-cold acetone to the resuspended pilins, vortexing for 1 min, and incubating at −20°C for 30 min. The proteins were collected by centrifugation for 20 min at 4°C at maximum speed in a microcentrifuge. The acetone was removed and the pellet dried for 10 min using a SpeedVac. The dry pellet was resuspended in 10 μl of formic acid, and once it was in solution, 90 μl of hexafluoro-2-propanol was added to completely solubilize the protein. An intact mass spectrum was obtained using a Q-TOF two-hybrid quadrupole time of flight mass spectrometer (Waters). The pilin solution was infused at 1 μl/min into the electrospray ionization source. Protein mass spectra were recorded in the range of *m/z* 800 to 3,000. The protein molecular weight profile was generated from the spectra by use of MaxEnt software (Waters).

Generation of a chimeric pilin gene cassette. The hybrid group V pilin-group III accessory gene construct was created using splicing by overlap extension (SOE) PCR (24). The $pilA_{III\text{-}tfpY}$ and $pilA_{V\text{-}tfpZ}$ constructs in pBADGr were used as templates to SOE $pilA_{V}$ to *tfpY*, allowing the amplification of the multiple cloning site from pBADGr with the genes for subsequent cloning. PCR primers designed to amplify $pilA_{V}$ from *P. aeruginosa* Pa281457, leaving a 3′ overhang complementary to the 5′ end of *P. aeruginosa* PA14 *tfpY*, were cartridge purified and were as follows: forward pBADGr (5′-TCTCTACTGTTTCTCCATACCCG) and reverse Pa281457 *pilA* + PA14 *tfpY* tail (5′-GAGGGGCTCTTTTCAGCATTAGCCTATTAGCGGCACTGAGCAGGAGCAAACT [the PA14 *tfpY* tail portion is underlined]). PCR primers designed to amplify *tfpY* from *P. aeruginosa* PA14, leaving a 5′ overhang complementary to the 3′ end of *P. aeruginosa* Pa281457 *pilA*, were cartridge purified and were as follows: forward PA14 *tfpY* + Pa281457 *pilA* tail (5′-AGTTTGCTCCTG

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
E. coli		
SM10	*thi-1 thr leu tonA lacY supE recARP42-2*-Tc^r::Mu; Km^r	51
DH5α	F$^-$ ϕ80d*lacZ*M15 (*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1* λ[συπ][[ρ]]$^-$	Invitrogen
P. aeruginosa		
1244	Group I T4P, used as a source for group I genes	10
PAO1	Group II T4P, used as a source for group III genes	26
PA14	Group III TFP, used as a source for group III genes	39
Pa5196	Group IV T4P, used as a source for group IV genes	29
Pa281457	Group V strain, used as a source for group V genes	29
Pa141123	Group III strain, used as source of pilin for mass spectrometry and to generate polyclonal antisera	29
Pa110594	Group V strain, used as a source of pilin to generate polyclonal antisera	29
Pa5325	Group V strain, used as a source of pilin for mass spectrometry	29
PAO1 NP	Transposon mutant 30458, Tn*5* IS*phoA*/hah insertion in *pilA* at nucleotide 165; has no pili (abbreviated NP)	26
PAO1 NP + $pilA_{II}$	NP mutant complemented with the PAO1 $pilA_{II}$ gene in pBADGr	This study
PAO1 NP + $pilA_{I}$	NP mutant complemented with the strain 1244 $pilA_{I}$ in pBADGr	This study
PAO1 NP + $pilA_{I}$-*tfpO*	NP mutant complemented with the strain 1244 $pilA_{I}$ and *tfpO* genes in pBADGr	This study
PAO1 NP + $pilA_{IV}$	NP mutant complemented with the strain Pa5196 $pilA_{IV}$ gene in pBADGr	This study
PAO1 NP + $pilA_{IV}$-*tfpX*	NP mutant complemented with the Pa5196 $pilA_{IV}$ and *tfpX* genes in pBADGr	This study
PAO1 NP + $pilA_{III}$	NP mutant complemented with the strain PA14 $pilA_{III}$ gene in pBADGr	This study
PAO1 NP + $pilA_{III}$-*tfpY*	NP mutant complemented with the strain PA14 $pilA_{III}$ and *tfpY* genes in pBADGr	This study
PAO1 NP + $pilA_{III}$-*tfpY#*	NP mutant complemented with the strain PA14 $pilA_{III}$ and *tfpY* genes in pBADGr; a premature stop codon at Leu59 in *tfpY* is indicated as #	This study
PAO1 NP + $pilA_{V}$	NP mutant complemented with the strain Pa281457 $pilA_{V}$ gene in pBADGr	This study
PAO1 NP + $pilA_{V}$-*tfpZ*	NP mutant complemented with the strain Pa281457 $pilA_{V}$ and *tfpZ* genes in pBADGr	This study
PAO1 NP + $pilA_{V}$-*tfpZ#*	NP mutant complemented with the strain Pa281457 $pilA_{V}$ and *tfpZ* genes in pBADGr; a premature stop codon at Leu58 in *tfpZ* indicated as #	This study
PAO1 NP *pilT*::*FRT*	NP mutant with an FRT disruption in the *pilT* gene	This study
PAO1 NP *pilT*::FRT + $pilA_{III}$	NP-*pilT* double mutant complemented with the strain PA14 $pilA_{III}$ gene in pBADGr	This study
PAO1 NP *pilT*::FRT + $pilA_{III}$-*tfpY*	NP-*pilT* double mutant complemented with the strain PA14 $pilA_{III}$ and *tfpY* genes in pBADGr	This study
PAO1 NP *pilT*::FRT + $pilA_{III}$-*tfpY#*	NP-*pilT* double mutant complemented with the strain PA14 $pilA_{III}$ and *tfpY* genes in pBADGr; a premature stop codon at Leu59 in *tfpY* indicated as #	This study
PAO1 NP *pilT*::FRT + $pilA_{V}$	NP-*pilT* double mutant complemented with the strain Pa281457 $pilA_{V}$ gene in pBADGr	This study
PAO1 NP *pilT*::FRT + $pilA_{V}$-*tfpZ*	NP-*pilT* double mutant complemented with with the strain Pa281457 $pilA_{V}$ and *tfpZ* genes in pBADGr	This study
PAO1 NP *pilT*::FRT + $pilA_{V}$-*tfpZ#*	NP-*pilT* double mutant complemented with the strain Pa281457 $pilA_{V}$ and *tfpZ* genes in pBADGr; a premature stop codon at Leu58 in *tfpZ* indicated as #	This study
PAO1 NP + $pilA_{V}$-*tfpY*	NP strain complemented with the strain Pa281457 $pilA_{V}$ gene and the *tfpY* gene from group III strain PA14 in pBADGr	This study
PA14 + pBADGr	PA14 strain carrying the pBADGr vector	This study
PA14 + $pilA_{III}$	PA14 strain complemented with the PA14 $pilA_{III}$ gene in pBADGr	This study
PA14 + $pilA_{III}$-*tfpY*	PA14 strain complemented with the PA14 $pilA_{III}$ and *tfpY* genes in pBADGr	This study
PA14 *tfpY*::FRT	PA14 strain with an FRT disruption in the *tfpY* gene	This study
PA14 *pilT*::FRT	PA14 strain with an FRT disruption in the *pilT* gene	This study
PA14 *tfpY*::FRT *pilT*::FRT	PA14 strain with FRT disruptions in the *tfpY* and pilT genes	This study
PA14 *tfpY*::FRT + pBADGr	PA14 strain carrying the pBADGr vector	This study
PA14 *tfpY*::FRT + $pilA_{III}$	PA14 *tfpY* mutant complemented with the PA14 $pilA_{III}$ gene cloned into pBADGr as above	This study
PA14 *tfpY*::FRT + $pilA_{III}$-*tfpY*	PA14 *tfpY* mutant complemented with the PA14 $pilA_{III}$ and *tfpY* genes cloned into pBADGr	This study

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant characteristic(s)	Reference or source
Plasmids		
pEX18Ap	Ap^r; *oriT*$^+$ *sacB*$^+$	23
pEX18Ap + *PA14pilB-pilA-tfpY-nadC*	3′ end of *pilB*, *pilA*, *tfpY*; 5′ end of *nadC* amplified from PA14 chromosomal DNA, digested with EcoRI and XbaI, and ligated into the vector's EcoRI and XbaI sites	This study
pEX18Ap + PA14 *pilB-pilA-tfpY-nadC*-SmaI	Site-directed mutation of base T117G to create a SmaI site in *tfpY*	This study
pEX18Ap + PA14 *pilBpilAtfpYnadC-GmFRT*	Gm-FRT insertion in the SmaI site of *tfpY*	This study
pEX18Ap + *pilT*::*GmFRT*	Gm-FRT insertion in the NruI site within *pilT*	This study
pPS856	Source of the FRT-flanked Gm resistance cassette (*aacC1*) used to disrupt *tfpY* and *pilT*	23
pFLP2	Source of the Flp recombinase used to excise the FRT-flanked Gm resistance cassette following mutagenesis	23
pBADGr	*ori araC-P*$_{BAD}$ *dhfr*::Gm^r *mob*$^+$	This study
pBADGr + *pilA*$_I$	*pilA*$_I$ from strain 1244 cloned into the EcoRI and HindIII sites as indicated in Materials and Methods	This study
pBADGr + *pilA*$_{II}$	*pilA*$_{II}$ from strain PAO1 cloned as above	This study
pBADGr + *pilA*$_{IV}$	*pilA*$_{IV}$ from strain Pa5196 cloned as above	This study
pBADGr + *pilA*$_{III}$	*pilA*$_{III}$ from strain PA14 cloned as above	This study
pBADGr + *pilA*$_{III}$-*tfpY*	*pilA*$_{III}$ and *tfpY* from PA14 cloned as above	This study
pBADGr + *pilA*$_{III}$-*tfpY#*	*pilA*$_{III}$ and *tfpY* from PA14 cloned as above; a premature stop codon at Leu59 is indicated as #	This study
pBADGr + *pilA*$_V$	*pilA*$_V$ from Pa281457 cloned as above	This study
pBADGr + *pilA*$_V$-*tfpZ*	*pilA*$_V$ and *tfpZ* from Pa281457 cloned as above	This study
pBADGr + *pilA*$_V$-*tfpZ#*	*pilA*$_V$ and *tfpZ* from Pa281457 cloned as above; a premature stop codon at Leu58 is indicated as #	This study
pBADGr + *pilA*$_V$-*tfpY*	*pilA*$_V$ from strain Pa281457 and *tfpY* from group III strain PA14 cloned together by SOE PCR (24) into the EcoRI and HindIII sites of pBADGr	This study

CTCAGTGCCGCTAATAGGCTAATGCTGAAAAGAGCCCCTC [the 1457 *pilA* tail portion is underlined]) and reverse pBADGr (5′-CGGCATG GGGTCAGGTGGGA). Total volumes were 50 μl and consisted of 2 μl of template DNA, 1 μl of 0.062 μM SOE primer, 1 μl of pBADGr primer (0.145 μM forward and 0.159 μM reverse), 5 μl of 10× PCR buffer, 10 μl Q solution (Qiagen), 0.5 μl 100 mM deoxynucleoside triphosphates, and 1 μl HotStar *Taq*. PCR consisted of a 15-min denaturation at 95°C followed by 30 cycles of 45 s at 95°C, 30 s at 55°C, and 2 min at 72°C, with a final extension of 7 min at 72°C and ending at 4°C. Primers were synthesized by Mobix, Hamilton, ON. Plasmids were isolated from *E. coli* DH5α by use of a QIAprep spin miniprep kit by following the manufacturer's instructions (Qiagen).

PCR products were separated in a 1% agarose gel and purified using a QIAquick gel purification kit by following the manufacturer's instructions (Qiagen), and DNA was resuspended in 50 μl of distilled H_2O. To SOE the two PCR products together, a PCR mixture consisting of 48 μl in total and containing 0.25 μl each of the two purified PCR products, 5 μl 10× PCR buffer, 10 μl of Q solution (Qiagen), 0.5 μl of 100 mM deoxynucleoside triphosphates, and 1 μl of HotStar *Taq* was made. One microliter each of forward and reverse pBADGr primers was added to the PCR mixture after the third cycle of denaturation-annealing-extension to bring the total volume to 50 μl. PCR consisted of a 15-min denaturation at 95°C followed by 3 cycles of 1 min at 95°C, 1 min at 50°C, and 2.5 min at 72°C, the addition of the primers, and 27 more cycles of the same temperatures/times, with a final extension of 7 min at 72°C and ending at 4°C. The SOE product, *pilA*$_{V\text{-}tfpY}$, was gel purified (Qiagen) and DNA was resuspended in 30 μl of distilled H_2O. The pBADGr vector and SOE product were each digested with EcoRI and HindIII, column purified (Qiagen), and ligated using standard methods.

Generation of a *tfpY* knockout mutant in PA14. A *tfpY* knockout mutant was created as described previously (48). Briefly, *tfpY* and flanking DNA were amplified by PCR using a forward primer (EcoRI restriction site) in the upstream gene *pilB* (5′-AAAGAATTCGGCTGGATCGGAGATGCCGAC GAACAG) and a reverse primer (XbaI restriction site) in the downstream gene *nadC* (5′-AAATCTAGAACCGCCACCCGCAGCCAGCACTACG) (restriction sites are underlined). The resulting PCR product was cloned into a suicide plasmid, pEX18Ap (23), and a SmaI site was introduced into *tfpY* at nucleotide 115 by changing T117 to G by site-directed mutagenesis (QuikChange; Stratagene). A Flp recombination target sequence (FRT)-flanked Gm resistance cassette was released from pPS856 (23) by use of SmaI and cloned into the suicide construct at the newly generated site. The resulting knockout construct was introduced into *E. coli* SM10 by electroporation. The knockout construct was then transferred to *P. aeruginosa* PA14 by biparental mating as described previously (7). After counterselection of *E. coli* SM10 on *Pseudomonas* isolation agar containing 30 mg/liter of Gm, exconjugants were replica plated onto LB agar containing either 30 mg/liter of Gm or 75 mg/liter of piperacillin. Colonies growing on Gm only were transformed with pFLP2 to excise the resistance cassette (23). Transformants were plated on 5% sucrose plates to counterselect the pFLP2 plasmid and then replica plated onto LB agar containing either 30 mg/liter of Gm, 200 mg/liter of carbenicillin, or no antibiotic. The genotypes of colonies growing only on LB agar were verified by PCR and DNA sequencing to confirm the disruption of *tfpY* by a single FRT site.

Generation of *pilT* and *tfpY-pilT* double mutants in PA14. A *pilT* knockout construct was generated by amplifying *pilT* and flanking DNA from the PAO1 chromosome by use of primers pilT1 (5′ **GGATCC**GGTGTTTTCCTTGTC CGA) and pilT2 (5′ **AAGCTT**GAATCCTAGACGCAGTTCC) (boldface indicates BamHI and HindIII sites, respectively) and cloning the product into pEX18Ap (23). A Gm-FRT cassette released from pPS856 (23) with SmaI was cloned into an NruI site within *pilT*. The resulting knockout construct was introduced into *E. coli* SM10 by electroporation and then transferred to *P. aeruginosa* PA14 wild type and PA14 *tfpY*::FRT by biparental mating as described previously (7). The Gm cassette was removed by introduction of the pFLP2 plasmid followed by curing of the plasmid by sucrose counterselection (23). Genotypes of the PA14 *pilT* knockout and PA14 *tfpY-pilT* double knockout mutants were verified by PCR and DNA sequencing to confirm the disruption of *pilT*.

RESULTS

Expression of *P. aeruginosa pilA* alleles in a PAO1 *pilA* mutant background. To examine potential functional differences among the five phylogenetically distinct *pilA* alleles in *P. aeruginosa* as well as to test possible contributions of the accessory

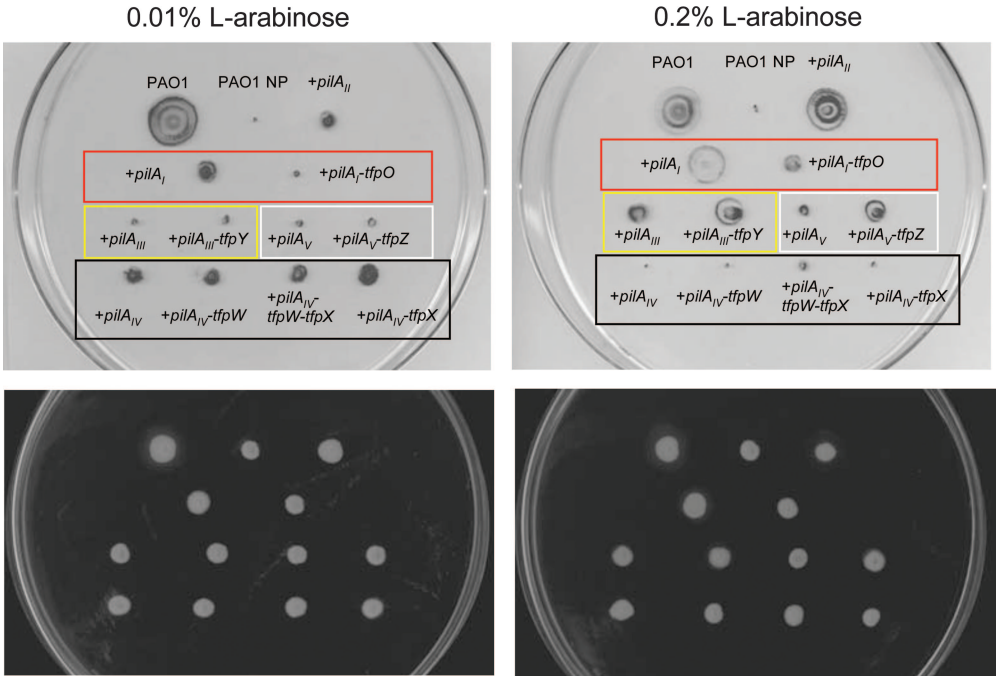

FIG. 1. Representative twitching motility of recombinant *P. aeruginosa* PAO1 NP strains. A group II strain PAO1 NP mutant was transformed with constructs expressing genes from group I strain 1244 (accessory gene *tfpO*) (boxed in red), group III strain PA14 (accessory gene *tfpY*) (boxed in yellow), group IV strain Pa5196 (accessory genes *tfpW* and *tfpX*) (boxed in black), and group V strain Pa281457 (accessory gene *tfpZ*) (boxed in white). The PAO1 wild type and the PAO1 NP mutant, both carrying the vector pBADGr, are positive and negative controls, respectively. Twitching motility was tested at two different arabinose concentrations, 0.01% and 0.2%; the cognate PAO1 pilin gene ($pilA_{II}$) restores wild-type motility at a 0.2% concentration of arabinose. The group I pilin gene complements the group II mutant to wild-type levels at 0.2% arabinose, but coexpression of the *tfpO* accessory gene at either arabinose concentration reduces motility. The group III and group V pilin genes do not complement well at either arabinose concentration tested, although motility is increased upon coexpression of their cognate accessory genes, *tfpY* and *tfpZ*, respectively, with 0.2% arabinose. The group IV pilin gene behaves aberrantly in that it complements the motility of PAO1 NP to the same extent as the cognate PAO1 pilin gene at 0.01% arabinose but not at 0.2%; this effect is independent of the *tfpW* and *tfpX* accessory genes. Below the twitching plates are growth controls showing that none of the observed differences in motility are due to growth defects.

genes to pilus function, we expressed the genes in a single genetic background. PAO1 is the most widely used and well-characterized laboratory strain of *P. aeruginosa*; therefore, we used a PAO1 *pilA* mutant (designated PAO1 NP, for *n*o *p*ili) as a host. As described in Materials and Methods, representative *pilA* genes from each group, either alone or in combination with their respective accessory genes, were cloned into pBADGr, an L-arabinose-inducible broad-host-range plasmid, and introduced into the NP strain. The level of L-arabinose supplementation required to restore wild-type twitching motility using the homologous group II PAO1 *pilA* gene ($PilA_{II}$) was determined to be 0.2% (wt/vol) (Fig. 1), and this concentration was used for complementation experiments.

Complementation of twitching motility by *P. aeruginosa pilA* and accessory genes. The ability of each of the five pilin alleles (designated with subscripts) to restore twitching motility to the PAO1 NP strain was tested. The $pilA_I$ and $pilA_{II}$ genes restored twitching motility to wild-type levels, while $pilA_{III}$ and $pilA_V$ only partially restored motility (Fig. 1). $pilA_{IV}$ was unable to restore motility to the NP strain when 0.2% L-arabinose was used to induce expression but did restore motility to the same extent as the cognate PAO1 gene when 0.01% L-arabinose was used. When the *pilA* genes were introduced with their cognate accessory genes, twitching motility was altered. Provision of $pilA_I$ together with *tfpO* caused a slight decrease in twitching motility compared with what was seen for $pilA_I$ alone (Fig. 1); similar results were reported previously by Smedley and colleagues (45). Cointroduction of $pilA_{III}$ with *tfpY*, $pilA_{IV}$ with *tfpX*, or $pilA_V$ with *tfpZ* increased twitching motility compared to what was seen for complementation with the *pilA* genes alone (Fig. 1). These data suggest that the pilin accessory genes *tfpX*, *tfpY*, and *tfpZ* affect pilus function. Because the group IV pilin behaved aberrantly with respect to the response to arabinose induction compared with pilins of the other four groups, further studies focused on the group III and V pilins.

Mass spectrometry analysis of $PilA_{III}$ and $PilA_V$. The enhancement of twitching motility in the PAO1 background in the presence of TfpY and TfpZ implied that the accessory proteins could play a role in pilin modification, assembly, or function. We noted previously that the group III and group V pilins migrate more rapidly on SDS-PAGE gels than would be expected based on their predicted masses. To determine whether these proteins were posttranslationally modified, pilins purified from wild-type strains 87141123 (group III) and 5325 (group V) were analyzed by mass spectrometry; these strains were selected because they are representative of their respective groups (29) and produce substantial amounts of surface pili, which facilitated the preparation of material for analysis. Figure 2 shows the intact mass spectra for these pilins;

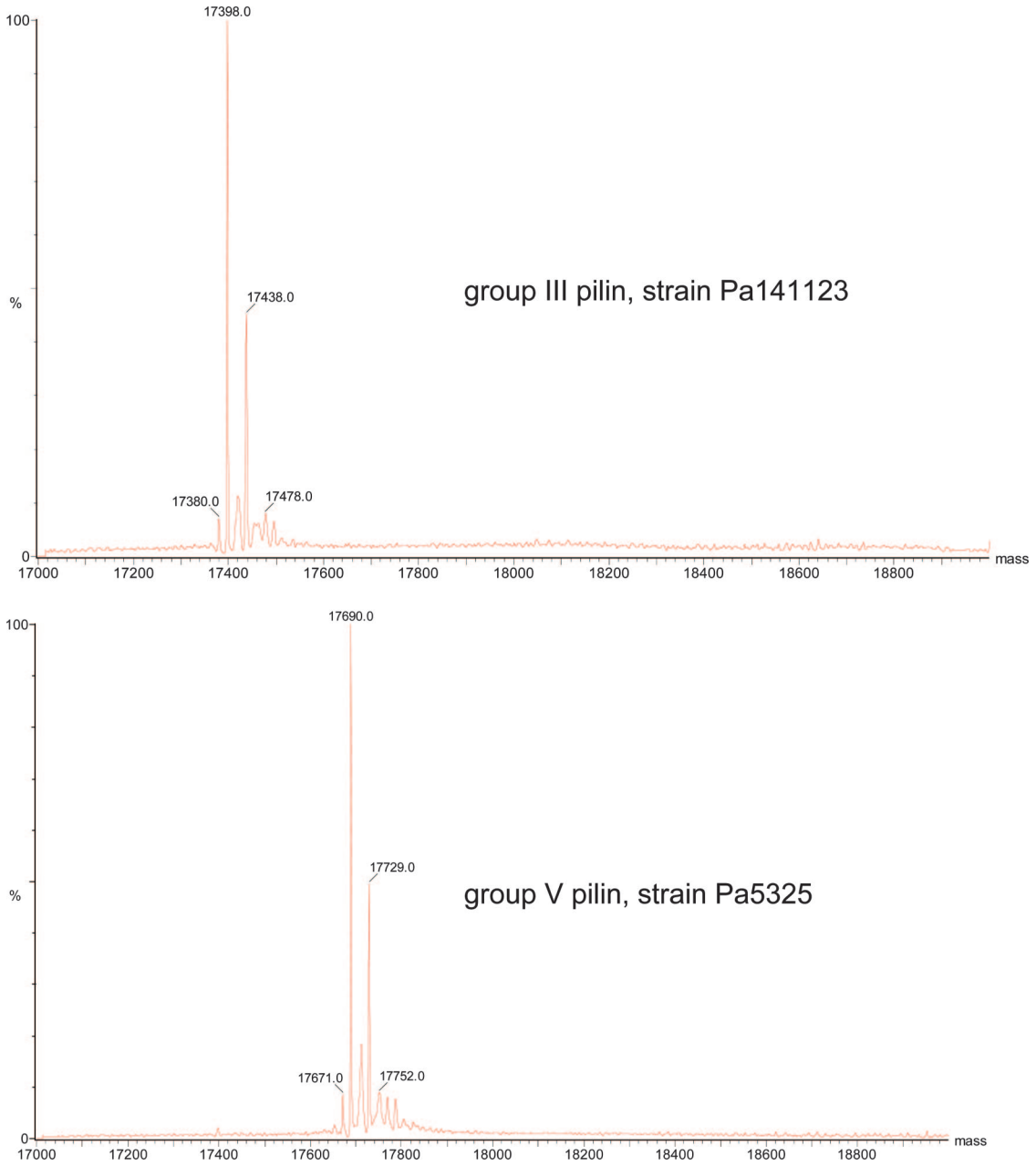

FIG. 2. Intact mass spectra of group III and group V pilins. The observed masses of 17,398 Da (group III pilin) and 17,696 Da (group V pilin) correspond to those predicted from the amino acid sequences (17,388 kDa and 17,705 kDa, respectively), showing that the pilins are not posttranslationally modified. The secondary peak is likely due to contaminating potassium or sodium adducts.

both have a mass corresponding to that predicted from their amino acid sequences, showing that they are not likely to be posttranslationally modified. A slightly heavier but less prominent peak, most likely due to salt contamination of the protein preparation, was observed in both spectra. Therefore, unlike what is the case for the TfpO and TfpW accessory proteins, the function of the TfpY and TfpZ proteins does not appear to be posttranslational modification of their cognate pilins.

TfpY and TfpZ increase levels of recoverable surface pili. The motility of recombinant strains expressing group III and V pilins was increased in the presence of the cognate accessory genes; to confirm this result, the complementation constructs containing $pilA_{\text{III-}tfpY}$ and $pilA_{\text{V-}tfpZ}$ were mutagenized to introduce a premature stop codon into each of the accessory genes. Twitching motility of the PAO1 NP mutant complemented with the pilin genes only, with the pilin and accessory genes, or with the pilin and mutant accessory genes was tested. Inactivation of *tfpY* and *tfpZ* caused a decrease in twitching motility to levels conferred by complementation with $pilA_{\text{III}}$ or $pilA_{\text{V}}$ alone, confirming that the accessory gene products are responsible for the observed increases in motility (Fig. 3).

TfpY and TfpZ could enhance twitching motility via in-

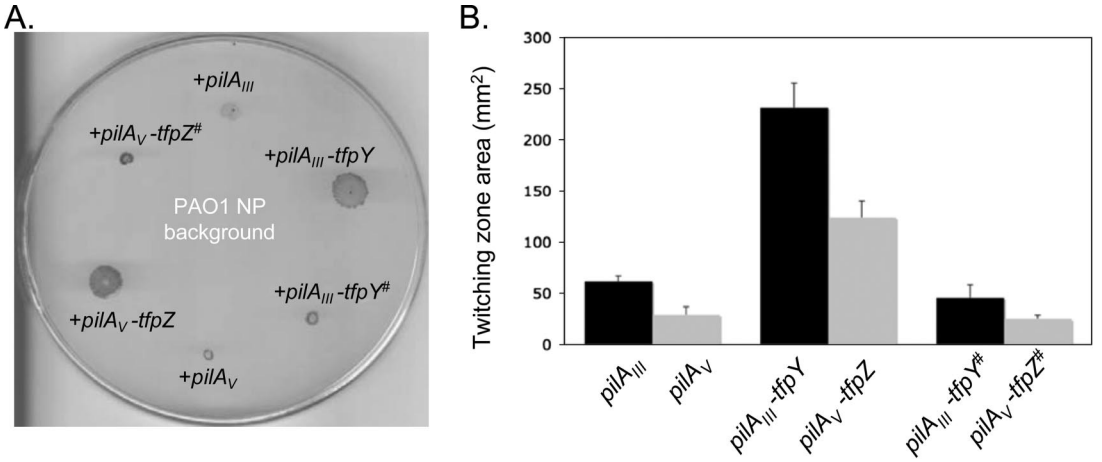

FIG. 3. Comparison of twitching zones in recombinant strains. (A) Twitching zones on 0.2% arabinose plates of the PAO1 NP strain expressing the indicated genes from strains PA14 (group III) and Pa281457 (group V). Each gene marked with a pound sign (#) contains a premature stop codon introduced by site-directed mutagenesis as outlined in Materials and Methods. (B) Quantitation of twitching zone areas (mm^2); average of 12 individual zones for each sample.

creased assembly of pili on the cell surface or by modulating total cellular pilin levels. To distinguish between these possibilities, we compared the levels of sheared surface pili and whole-cell pilin levels among the recombinant strains. Figure 4 shows that the complemented strains have similar levels of pilins in whole-cell lysates, but strains lacking the accessory genes (either by deletion or disruption via premature stop codon) had fewer recoverable pili on the surfaces of cells. This result suggests that the impaired twitching motility of strains lacking functional pilin accessory proteins is due to decreased surface pilus levels.

Conservation of other components of the T4P system among *P. aeruginosa* strains with group I to group IV pilin alleles. The inability of group III and V pilins to restore twitching motility to wild-type levels in the PAO1 background was unexpected, since these genes were sourced directly from other *P. aeruginosa* strains and this organism has previously been reported to express heterologous pilins from other bacteria (15, 25, 50). Since reduced twitching motility in the group II background strain PAO1 appeared to arise from decreased surface piliation and therefore from potential assembly defects, we asked whether other components involved in pilus assembly were

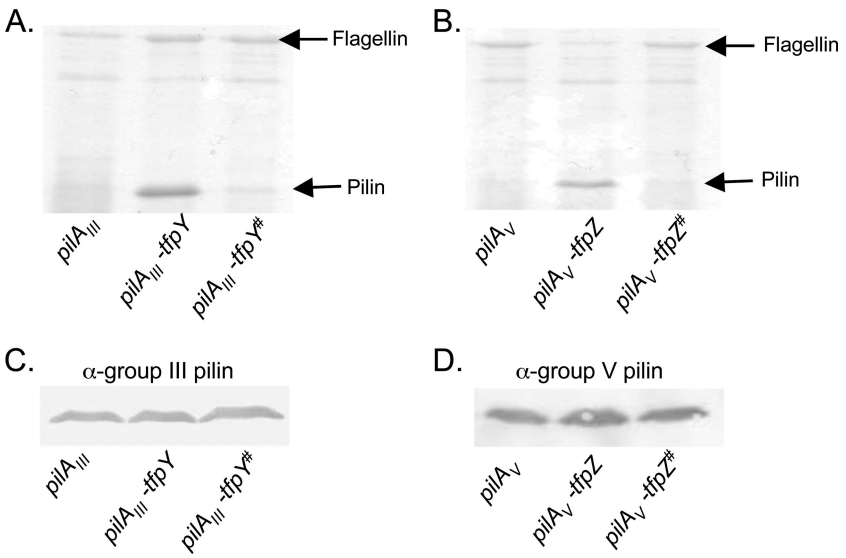

FIG. 4. Loss of accessory proteins reduces surface piliation but not whole-cell pilin pools. Representative SDS-PAGE gels showing that the lack of the pilin accessory genes reduces surface piliation in PAO1 recombinant strains expressing group III PA14 (A) and group V Pa281457 (B) genes. Pilins are ~15 kDa, and flagellins used as a loading control are ~50 kDa. Pilin levels were normalized to flagellin levels by densitometry using ImageJ (Scion). The presence of the accessory protein resulted in a 3-fold increase (group III, TfpY) or a 10-fold increase (group V, TfpZ) in the amount of surface pili recovered. Western blots of whole-cell lysates from PAO1 recombinant strains obtained using α group III pilin (C) or α group V pilin (D) sera show that the lack of surface piliation is not due to decreased pilin levels in whole-cell pools. Each gene marked with a pound sign (#) contains a premature stop codon introduced by site-directed mutagenesis as outlined in Materials and Methods.

TABLE 2. Conservation of type IV pilus accessory and assembly proteins among *P. aeruginosa* strains

Type IV protein	Presence in, or % similarity to respective PAO1 protein for, indicated *P. aeruginosa* strain (*pilA* allele group; accession no. or identification)							Reference
	2192 (group I; AAKW00000000)	LES (group I; Sanger)	PAO1 (group II; AE004091)	PACS2 (group II; AAQW00000000)	PA14 (group III; CP000438)	C3719 (group III; AAKV00000000)	PA7 (group IV; AAQE00000000)	
Accessory protein[a]								
TfpO	+	+	−	−	−	−	−	9
TfpWX	−	−	−	−	−	−	+	29
TfpY	−	−	−	−	+	+	−	29
TfpZ	−	−	−	−	−	−	−	29
Assembly protein[b]								
PilA	53	60	100	75	45	45	47	29
PilB	94	94	100	98	96	96	94	36
PilC	93	93	100	98	94	95	94	36
PilD	99	99	100	99	98	99	97	36
PilT	100	100	100	100	100	100	100	12
PilU	100	100	100	100	100	100	100	52
PilM	100	100	100	100	100	100	100	33
PilN	100	100	100	100	96	95	100	33
PilO	100	100	100	100	97	97	99	33
PilP	100	100	100	100	96	96	99	33
PilQ	98	98	100	99	96	97	98	33
PilF	99	99	100	99	99	99	97	49
FimV	100	99	100	99	98	99	94	43
PilZ	100	100	100	100	100	100	100	3
FimT	99	99	100	99	49	49	81	1
FimU	100	100	100	100	65	65	94	1
PilV	100	100	100	100	75	75	93	2
PilW	98	100	100	99	68	68	93	4
PilX	100	100	100	100	75	75	94	4
PilY1	95	95	100	95	69	69	94	4
PilY2	100	99	100	100	51	51	88	4
PilE	100	100	100	100	61	61	97	4

[a] Proteins encoded immediately downstream of *pilA*.

[b] Proteins that have been shown by mutagenesis to be required for pilus assembly in *P. aeruginosa*; in many cases, their specific functions are not known. The percentages of similarity at the amino acid level to the respective PAO1 protein, as determined by pairwise comparisons using BLAST, are shown.

conserved among *P. aeruginosa* strains. We first analyzed the currently available genome sequences of *P. aeruginosa* for the characteristic accessory genes associated with each pilin allele in order to determine the pilin type of each strain (29). There are two genomes each of strains with group I (LES, 2192), group II (PAO1, PACS2), and group III (PA14, C3719) pilins and one group IV pilin strain (PA7) currently available. No group V pilin gene-containing strain has yet been sequenced. After identifying the pilin allele, we performed BLASTp or tBLASTn searches using the amino acid sequences of pilus assembly proteins from PAO1 as in silico probes for each of the other genomes. A summary of this analysis is shown in Table 2. The major components of the *P. aeruginosa* T4P system, including the prepilin peptidase PilD, the membrane protein PilC, the motor proteins PilB, PilT, and PilU, and the assembly factors PilM, PilN, PilO, PilP, PilQ, PilF, and FimV are highly conserved (greater than 90% amino acid similarity). FimT, FimU, PilV, PilW, PilX, PilY1, PilY2, and PilE are essentially identical between strains carrying group I and group II pilin genes, while these gene products are less similar to those of the PAO1 lab strain in PA7, a strain carrying a group IV pilin, and even further divergent from PAO1 in strains that have group III pilins (Table 2). However, the predicted minor pilin gene products in the two group III strains sequenced to date (PA14 and C3719) are identical.

Expression of heterologous pilins in a retraction-deficient background. For *Neisseria*, it has been shown previously that decreased surface piliation in some mutants can arise from alterations in the dynamics of pilus retraction mediated by the PilT retraction ATPase. This phenotype can be distinguished from one in which there is a complete inability to assemble pili by inactivating PilT and testing for the restoration of surface piliation (8, 54, 55). To determine if the decrease in surface piliation seen in recombinant *P. aeruginosa* strains expressing group III or group V pilins was due to altered retraction dynamics, we introduced the constructs into a PAO1 *pilA-pilT* double mutant and examined surface pilus expression (Fig. 5). In this background, all of the strains expressing pilins of a particular group expressed similar levels of surface pili, showing that the observed reduction in motility and surface piliation in the *pilT*-replete background is due to altered pilus retraction dynamics. The pilins isolated from the double mutant strains were similar to one another in mass (as determined by migration on SDS-PAGE gels) regardless of the presence of the accessory proteins, providing additional evidence that the accessory proteins do not posttranslationally modify their associated pilins in either the native or recombinant backgrounds. Although the trend was the same for both group III and group V recombinant strains, the increase in surface piliation in the absence of *pilT* was more pronounced for group V (Fig. 5).

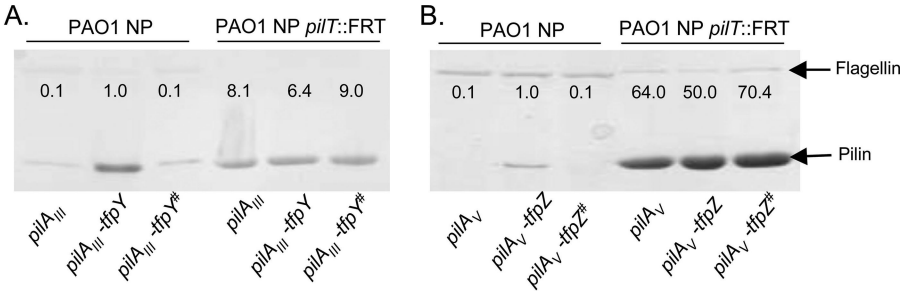

FIG. 5. Recovery of surface pili in a *pilT* mutant background. Representative SDS-PAGE gels showing that the expression of the pilin gene cassettes from PA14 (group III [A]) and Pa281457 (group V [B]) strains in a PAO1 NP-*pilT* double mutant resulted in the expression of large amounts of surface pili regardless of the presence of the pilin accessory gene (pound sign indicates a premature stop codon). Pilin levels were normalized to flagellin levels in each lane by densitometry using ImageJ (Scion). Note that the samples prepared from the PAO1 NP-*pilT* background were diluted 10-fold compared to those from the PAO1 NP background due to the large amounts of pilin present in these samples. The change in the amount of surface pilin recovered from each strain, relative to what was seen for the NP strain complemented with both pilin and accessory genes, is indicated in each lane.

Analysis of a *tfpY* knockout mutant in PA14. Since the above analyses examined the function of the accessory genes in recombinant strains, it was important to examine their role in the native background. A *tfpY* knockout mutant of group III strain PA14 was analyzed for twitching motility, surface pili, and whole-cell pilin levels. Phenotypes of the knockout mutant recapitulated those observed for the recombinant strains; twitching motility was decreased and the level of recoverable surface pili was reduced compared with what was seen for the wild type (Fig. 6), but the amount of pilin in the whole-cell lysates was similar to that for the PA14 wild-type strain (data not shown). Complementation of the *tfpY* mutant with the

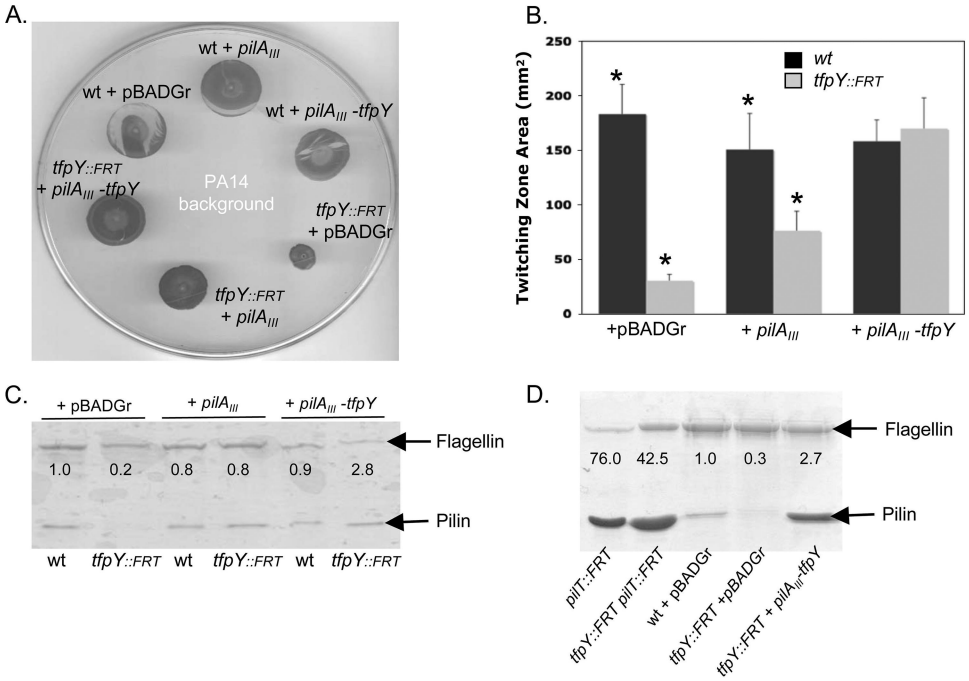

FIG. 6. Surface piliation and twitching is reduced in a PA14 *tfpY* knockout. (A) Representative plate showing that twitching motility is reduced in a *tfpY* mutant compared with the wild type (wt) but can be complemented back to wild-type levels in the presence of $pilA_{\text{III-}tfpY}$. (B) Average area in mm^2 of a minimum of six twitching zones per strain. Asterisks indicate significant differences ($P \leq 0.05$; Student's *t* test) between the motilities of wild-type and mutant strains. (C) SDS-PAGE gel of sheared surface proteins from the PA14 wild type and the PA14 *tfpY*::FRT mutant complemented with pBADGr, $pilA_{\text{III}}$, or $pilA_{\text{III-}tfpY}$. In the absence of TfpY surface piliation is reduced; complementation of the *tfpY*::FRT mutant with $pilA_{\text{III}}$ alone results in increased surface piliation, although motility does not return to wild-type levels as shown in panels A and B. Pilin levels were normalized to flagellin levels by densitometry using ImageJ (Scion), and the change in the amount of surface pilin recovered relative to what was found for the wild-type control is shown in each lane. (D) SDS-PAGE gel of sheared surface proteins. Although surface piliation of a *tfpY* mutant is low, it is recovered in the *tfpY-pilT* double mutant, although not to the same level as a *pilT* single mutant. Pilin levels were normalized to flagellin levels by densitometry using ImageJ (Scion). Note that the *pilT* and *tfpY-pilT* mutant samples have been diluted fivefold relative to the other samples due to the large amount of surface pili expressed by these strains. The change in the amount of surface pilin recovered relative to the wild type is shown in each lane.

pilA$_{III\text{-}tfpY}$ construct restored surface pilin levels to those of the wild type complemented with the same construct. Interestingly, complementation of the *tfpY* mutant with *pilA*$_{III}$ expressed from the arabinose-inducible promoter increased motility compared with what was seen for the *tfpY* mutant that has only a single chromosomal copy of *pilA* (Fig. 6), though not to wild-type levels, even though the amount of recovered surface pilin was similar. These results show that overexpression of PilA$_{III}$ in either the recombinant or mutant strains lacking TfpY is not sufficient to restore motility to wild-type levels and that TfpY is required for normal twitching motility and surface piliation in PA14. To ascertain whether pilus retraction dynamics were affected in the PA14 *tfpY* mutant, a *tfpY-pilT* double mutant was generated. The double mutant expressed large amounts of recoverable surface pili, approximately 250-fold more than the *tfpY* single mutant (Fig. 6D, lane 4) but only about half of the amount of a PA14 *pilT* single mutant (Fig. 6D, lanes 1 and 2). These data suggest that TfpY affects pilus retraction dynamics in the PA14 background and that it may also influence assembly in its native background.

The accessory proteins are specific for their cognate pilins. In all cases that we have examined to date (reference 29 and L. L. Burrows, unpublished data), the accessory genes are invariantly associated with a specific pilin allele (i.e., *tfpX* with *pilA*$_{IV}$, *tfpY* with *pilA*$_{III}$, and *tfpZ* with *pilA*$_{V}$). To determine whether the accessory proteins are functionally interchangeable, a chimera containing the pilin gene from the group V strain Pa281457 and the accessory gene *tfpY* from the group III strain PA14 was examined for its ability to complement twitching motility and surface piliation in the PAO1 NP background. Although the PAO1 NP-plus-*pilA*$_{V}$-*tfpY* strain was able to twitch, the size of the twitching zone was similar to that of the strain complemented with *pilA*$_{V}$ alone (Fig. 7). The chimera expressed levels of pilin in whole-cell lysates similar to those seen for strains expressing *pilA*$_{V}$ alone or with *tfpZ*, but the level of recoverable surface pili was similar to that of the strain lacking the accessory protein (Fig. 7). These data suggest that the group III accessory protein TfpY is incompatible with the group V pilin and that the accessory proteins therefore are specific for their cognate pilins.

DISCUSSION

In our previous study of *P. aeruginosa* pilin diversity, we defined three new pilin alleles that were found in ~25% of the genetically distinct environmental, clinical, and cystic fibrosis samples that were tested (29). Strain PA14, which expresses group III pilins, was recently shown to be the most common clonal strain in the *P. aeruginosa* population, representing 7% of 244 strains tested from a wide range of environments (53). Together, those studies suggest that the group III, IV, and V pilin alleles are more common in *P. aeruginosa* than was previously appreciated. The striking linkage between the group III and V pilin genes and their accessory genes, *tfpY* and *tfpZ*, respectively, in strains of different genotypes could be fortuitous due to their proximity in the chromosome or indicative of a functional relationship. Here we showed that the accessory proteins modulate pilus retraction dynamics, as the loss of these proteins decreased surface pili and twitching motility but did not affect pilin assembly per se or alter the whole-cell pilin pools. These observations, together with our data showing that TfpY is not compatible with the group V pilin PilA$_{V}$, lead us to conclude that there is a functional explanation for the observed linkage between specific pilins and their cognate accessory proteins.

To our knowledge, only two type IV pilin accessory genes of this type have been examined in the literature. The accessory gene product PilB from *E. corrodens* (31% protein sequence identity to TfpZ, concentrated in the predicted N-terminal transmembrane regions) was shown to be required for twitching motility and possibly for pilus assembly; however, its specific function is unknown (46). On the other hand, *D. nodosus* accessory gene product FimB (32% protein sequence identity to TfpZ) was found not to be required for pilus biogenesis; again, its function is unknown (28). Although these reports seem contradictory, our findings are consistent with both; for example, loss of the TfpY accessory protein causes decreased twitching motility in both native (PA14) and recombinant (PAO1) backgrounds but does not completely abrogate pilus assembly.

When the pilins from groups III and V were expressed in a PAO1 background, we were initially surprised to find that they were unable to complement twitching motility and surface piliation to levels seen with the homologous control, since there have been previous reports of successful expression of heterologous type IV pilins in *P. aeruginosa* (15, 25, 50, 54). We showed that the group III and V pilins were stably expressed in the absence of their associated accessory proteins and that the observed motility defects were occurring at the level of pilus assembly/disassembly. This hypothesis was supported by the recovery of large amounts of surface pili upon expression of the group III and V pilins both with and without their cognate accessory proteins in a *pilA-pilT* double mutant, showing that (i) the T4P assembly machinery readily accommodates pilins of diverse sequences with similar efficiencies and (ii) the reduced surface piliation and motility in accessory protein-deficient strains is not due to assembly defects per se. The high conservation among components of the T4P assembly system in *P. aeruginosa* strains expressing various *pilA* alleles (Table 2) is consistent with the concept that most components of the system are insensitive to differences in pilin primary sequence. Although type IV pilin sequences can be quite divergent, comparison of the structures of those solved to date shows that they have similar architectures (5, 13, 16, 21, 27, 37, 40), supporting the idea that they are likely to be functionally compatible with conserved components of the T4P machinery. The loss of TfpY in the PA14 *pilT* background caused a ~40% reduction in the amount of surface pilin recovered (Fig. 6D), suggesting that the accessory proteins may also have some role in promoting pilus assembly.

The extent of twitching motility observed and the amount of recoverable surface pili on a specific strain are determined not only by the ability to assemble a pilus but also by the crucial balance between rates of pilus extension versus retraction (54–56). Since the process of pilus assembly appeared to be independent of both pilin sequence and the presence/absence of the accessory proteins, the substantial decrease in surface piliation observed in the absence of the accessory proteins suggests that perhaps the rate of assembly of heterologous pilins may be lower than that of homologous pilins, resulting in a net

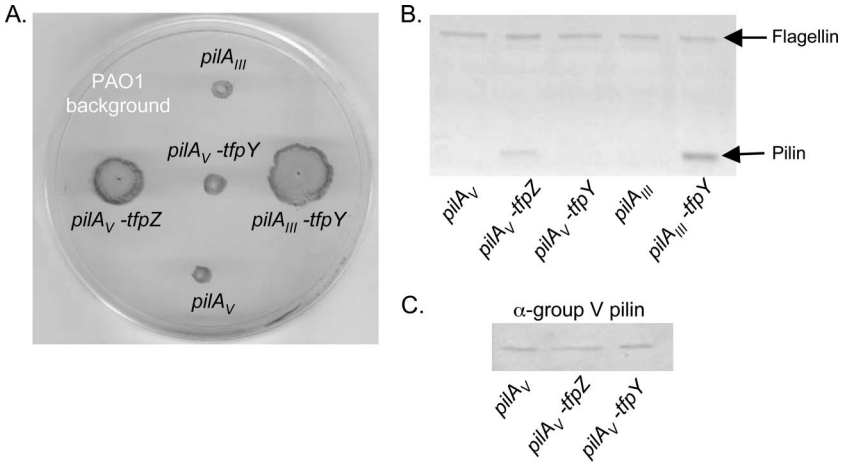

FIG. 7. Complementation of PAO1 NP with a group V pilin-group III accessory gene chimera. The group III PA14 accessory protein TfpY cannot replace the function of the group V Pa281457 accessory protein TfpZ. (A) Twitching motility in the PAO1 NP recombinant strain is similar to that seen for the PAO1 NP recombinant strains lacking a functional accessory protein. (B) Representative SDS-PAGE gel showing that surface piliation is reduced 10-fold in the strain expressing the chimera (lane 3) compared to that expressing the original $pilA_{V\text{-}tfpZ}$ cassette (lane 2) and similar to the level obtained by the expression of $pilA_V$ alone (lane 1). Pilin levels were normalized to flagellin levels by densitometry using ImageJ (Scion). (C) Whole-cell lysates probed with α group V pilin antisera show that the reduction of surface piliation in the chimeric strain is not due to differences in whole-cell pilin levels. The flagellin (~50 kDa) serves as a loading control.

increase in PilT-mediated retraction. Since PilT proteins are identical for all *P. aeruginosa* strains examined to date (Table 2), it is unlikely to be directly involved in pilin recognition. Although the rate of pilus retraction has been estimated using optical techniques to be approximately 1,000 to 1,500 subunits per second (31, 35, 44), it has not yet been possible to measure the rate of pilus extension due to the inherent flexibility and therefore the nonlinearity of the fibers.

Of the factors involved in T4P assembly that have been identified to date, only the minor pilins exhibit notable differences in sequence between strains with unrelated pilin alleles (Table 2). Interestingly, group I strains 2192 and LES have minor pilins that are 95 to 100% identical at the amino acid level to those of group II strain PAO1 (Table 2), and the $pilA_I$ gene from strain 1244 was able to complement PAO1 NP to the same extent as its cognate group II pilin. Similarly, the minor pilins of a group IV strain are similar (88 to 97%) to those of PAO1, with the exception of FimT (81%), and the $pilA_{IV}$ gene restored the motility of PAO1 NP to levels commensurate with the homologous PAO1 pilin gene (albeit only at lower arabinose concentrations, for reasons not yet clear). In contrast, the minor pilins from the group III strains PA14 and C3719 (no group V genome is yet available) have substantially less similarity to those of PAO1 (ranging from 49 to 75%) (Table 2) but are identical to one another. The minor pilins are important for the control of pilus assembly, although their specific functions are not yet understood (1, 2, 4, 55). Studies with *Neisseria* showed that at least one minor pilin can be incorporated into surface-exposed T4P and thereby influence pilus properties (22). We speculate that reduced compatibility between horizontally acquired heterologous major pilins and the host strain's set of minor pilins may slow the rate of pilus assembly and thereby cause the observed net retraction of heterologous pili mediated by PilT. However, because the inactivation of *tfpY* in PA14 also results in assembly defects (detectable in the *pilT* background) and altered retraction dynamics, it is possible that the pilins of groups III and V have unusual properties; for example, they are larger than those of groups I, II, and IV (29). Such differences could result in a reduced rate of assembly compared with those of other pilins and therefore a net increase in retraction in the absence of their cognate accessory protein. There are minor differences in the sequences of the conserved PilB assembly ATPase among different groups (Table 2), but they are localized to the N termini and are more likely to reflect past recombination events in the 5′ end of the gene, which is closest to the divergently transcribed *pilA* gene, rather than functional differences (29). Regardless of the underlying mechanism, the accessory proteins characterized in this study appear to modulate the balance between pilus assembly and disassembly, allowing assembled pili to remain on the cell surface long enough to allow pilus tip attachment and therefore successful twitching motility.

Our examination of a chimeric $pilA_{V\text{-}tfpY}$ construct showed that the accessory proteins are specific for their cognate pilins. Both TfpY and TfpZ are predicted to have short cytoplasmic N termini, three transmembrane domains, and periplasmic C termini consisting of ~132 (TfpY) and ~153 (TfpZ) residues. This orientation resembles that of the pilins, which are retained in the inner membrane prior to assembly by their hydrophobic N termini, with their C-terminal domains in the periplasm. The amino acid sequence similarity between TfpY and TfpZ is ~50%, restricted mainly to the membrane-spanning domains. Therefore, the divergent sequences of the C termini of the accessory proteins are likely to dictate pilin specificity, particularly since it is the C termini of the pilins that are most variable (38). Future studies will be aimed at determining whether there are direct interactions between the pilins and their associated accessory proteins.

As antigenic surface structures exposed to the environment, T4P are subject to evolutionary selection for variation, which can occur by point mutation, by intragenic recombination of

silent cassettes into active loci (as in *Neisseria*), by posttranslational modifications, and by horizontal gene transfer between species (6, 18, 29, 32, 38, 47). The observed linkage between specific pilin and accessory genes in *P. aeruginosa* appears to be a consequence of evolutionary selection for the improved function of T4P. The *tfpO* gene product encoded in the group I pilin cassette enhances virulence by posttranslational glycosylation of its specific pilin substrate and functions in any genetic background expressing a suitable O-antigen glycan (9, 45). The TfpY and TfpZ proteins characterized here appear to have been retained in the pilin cassette because they increase surface piliation and motility, likely improving the chances that their host strain could successfully colonize a desired environmental niche.

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